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(57) Abstract

The present invention relates generally to a method of characterising DNA. More particularly, the present invention contemplates a method of detecting base changes in DNA by determining the optical density at one or more temperatures. Particularly, the method utilises the differential optical densities of single-stranded DNA (ssDNA) and double stranded DNA (dsDNA) preferably to characterise the length and denaturation and renaturation temperatures of melting domains. Accordingly there is provided a method of characterising DNA including: providing a source of DNA; subjecting the DNA to a range of temperatures sufficient to cause a portion of the DNA to denature or to renature; and determining the optical density of the DNA within the range of temperatures.

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METHOD FOR ANALYSIS OF DNA

The present invention relates generally to a method of characterising DNA. More particularly, the present invention contemplates a method of detecting base changes in DNA by determining the optical density at one or more temperatures. Particularly, the method utilises the differential optical densities of single-stranded DNA (ssDNA) and double stranded DNA (dsDNA) preferably to characterise the length and denaturation and renaturation temperatures of melting domains.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises" is not intended to exclude other additives or components or integers.

Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have become increasingly popular as methods for detecting differences as small as single base mutations between samples of PCR-amplified DNA (for example, Barbetti *et al* 1992, Hovig *et al* 1991). These techniques rely on the principle that the electrophoretic mobility of the DNA is greatly decreased as double-stranded DNA (dsDNA) denatures in sequence-dependent melting domains along a linear temperature or denaturant concentration gradient. Other techniques which are utilised for the detection of DNA mutations include single-stranded conformational polymorphisms (SSCP-PCR) (for example, David *et al* 1993) [*Orita et al, 1989*] and chemical cleavage (Fodde and Losekoot 1994).

However, electrophoretic limitations of DGGE and TGGE make the detection of polymorphisms in regions other than the least stable melting domain difficult and fragments analysed using gradient gel electrophoresis must be less than 2kb. Denaturing gradient gel blots (Gray 1992) or the modification of PCR-amplified fragments by restriction enzymes or GC-clamps (Abrams and Stanton, 1992); improves resolution of polymorphic melting-domains. Multiple gels, each with different domain-specific denaturing conditions (Hovig et al 1991) or two-dimensional electrophoresis (Barbetti et al 1992) further improve the detection of polymorphic melting-domains.

These methods of detection often require substantial technical expertise, excessive handling of samples, and complicated equipment such as electrophoresis equipment. The results are not rapidly obtained and often are dependent on many parameters necessary for establishment of the technique such as correct gel conditions, pre-treatment of the DNA and expertise in analysis of the gels thereby reducing the reliability and reproducibility of the results.

It is an object of the present invention to overcome or at least alleviate some of the problems of the prior art and/or provide an alternative to the prior art methods.

Accordingly, one aspect of the present invention provides a method of characterising DNA including:

providing a source of DNA;

subjecting the DNA to a pre-determined temperature sufficient to cause a portion of the DNA to denature or to renature; and

determining the optical density of the DNA at the pre-determined temperature.

There are at least three things that govern the temperature (or denaturant concentration) at which dsDNA denatures or renatures; the sequence of each strand, the intra-molecular bond stability and base-stacking between successive turns within the double-stranded helix. (Abrams and Stanton [1992]; Rabinovich et al [1988]). The term *DNA melting* refers to the thermal or chemical denaturation of dsDNA. During denaturation the double helix melts at a denaturation temperature (T_m) to form two single-stranded molecules as the hydrogen bonds that link complimentary bases across the helix are disrupted. The segments of dsDNA that become single-stranded as the denaturation thresholds are exceeded are commonly referred to as *melting domains*. Conversely, as the temperature falls below the denaturation threshold, the DNA may be renatured.

Preferably the pre-determined temperature for measuring the optical density is a denaturation temperature indicating a melting domain. In a further

aspect of the present invention, there is provided a method of characterizing DNA including:

providing a source of DNA;

subjecting the DNA to one or more temperatures, and

determining the optical density of the DNA at each temperature.

Spectrophotometric typing of DNA in the present invention may characterise dsDNA with a defined length by determining the denaturation or renaturation temperature and length of melting-domains. A melting-domain within dsDNA denatures as its threshold temperature is exceeded. The denaturation-temperature is governed by complex intermolecular and intramolecular interactions, these interactions are largely determined by the composition and order of nucleotides within the dsDNA. The optical properties of DNA change as it denatures. Single-stranded DNA in aqueous solution absorbs approximately 37% more light in the UV range of the spectrum than dsDNA with the same nucleotide content (Fielder 1982). The optical density increases as denaturation occurs, producing discrete, quantifiable changes in absorbance which are directly proportional to the length of disassociated strands. Conversely, the optical density will decrease as renaturation occurs.

This method of the present invention which includes characterising the DNA may include determining base changes in the DNA to determine length of melting domains and/or the denaturation or renaturation temperatures of the individual samples.

The DNA samples may be double-stranded DNA and preferably the DNA sample is in an aqueous solution such as water, or phosphate buffered saline (PBS). The source of DNA may derive from any source including a plasmid, bacteriophage such as lambda, T2 or T4, cDNA or genomic DNA. The DNA may be reduced in length by any method including chemical or physical means such as digestion with restriction enzymes or by shearing by physical forces. The DNA may also undergo a purification step such as by electrophoresis or by chromatography so that all DNA tested is substantially the same size. Preferably the DNA is less than about 2kb however, any length of DNA can be used.

DNA amplified by the polymerase chain reaction (PCR) may also be a source of DNA. PCR may be conducted in a separate vessel such as microfuge tube or it may be conducted directly within a cuvette used to measure the optical density. The amount of DNA for characterisation using a 300µl cuvette may be synthesised in any efficient 50µl polymerase chain reaction. The single and unincorporated nucleotides present following PCR amplification of dsDNA may have no effect on the measurement of the changes in optical density.

Where the DNA is genomic DNA and isolated from cells or tissue, the DNA sample may undergo initial purification and identification to locate the gene of interest before the DNA is subjected to the method of the present invention for further characterisation. The purification step may also yield fragments of the same length or size.

The determination of optical density may be a change in the absorbance at a predetermined wavelength. The change in absorbance may be standardised and measured as a ratio of the change in absorbance and a change in temperature over a varying temperature range.

The optical density may be a measure of the absorbance and may be measured in a spectrophotometer including a centrifugal spectrophotometer or a conventional spectrophotometer. Other methods of measuring changes in optical density include incorporation of interchelating florescent agents such as ethidium bromide and a commonly used dye from Hoechts could be used to quantify the length of DNA that becomes single stranded as dsDNA denatures. Both agents destabilize the helix to some degree.

Preferably the wavelength for measuring optical density is in the range of about 250 to 300 nm and most preferably the wavelength is about 260 nm.

Centrifugal spectrophotometry may be computerised so as to improve the handling and time efficient characterisation of DNA. The use of a centrifugal spectrophotometer allows for automation of the method and thereby provides for the capacity of analysing large quantities of samples.

In a preferred form of the invention optical density of the sample is read at varying temperatures. The temperature range may include at least one temperature at which a portion of the DNA sample denatures (denaturing temperature) or renatures and this is generally associated with the sequence of each strand, the intramolecular bond stability and the base stacking between successive turns within the double-stranded helix. Preferably the temperature range includes several denaturing temperatures indicating several melting domains.

The temperature at which the DNA denatures may indicate at least one melting domain in the DNA where the DNA sample is read at one temperature, only a portion of the DNA may be denatured. Preferably, the temperature range will include more than one denaturing temperature indicating more than one melting domain.

By measuring the optical density at any one pre-determined temperature sufficient to cause denaturation of a portion of the DNA, differences in samples may indicate differences in denaturing temperature and lengths of melting domains.

The temperature range may be varied in a continuous manner or in a stepwise manner incorporating several ranges. Preferably, the temperature is increased at 1°C/min. The temperature may be applied by any method including the use of a heat block, hot water bath or hot air.

A preferred aspect of the present invention further includes increasing the yield of homoduplex molecules following PCR amplification. Accordingly, the present invention provides a method of characterising DNA including:

providing a source of DNA by PCR amplification;

subjecting the PCR amplification to conditions favouring homoduplex DNA molecule formation;

subjecting the DNA to one or more temperatures; and

determining optical density of the DNA at each temperature.

The PCR-amplification of heterozygous alleles in nuclear DNA produce four different types of molecules. Two molecules with perfect complementarity, these are referred to as *homoduplex* molecules and two *heteroduplex*

molecules from annealing of the near-complimentary strands amplified from the different alleles, (Sheffield et al 1990) resulting in complicated thermal denaturing profiles.

Increasing the yield of homoduplex molecules simplifies STOP DNA analysis of heterozygous alleles. This is preferably achieved using a primer annealing protocol adapted from direct DNA sequencing [*Roberts et al, 1991*]. On completion of the last PCR-extension (usually at 72°C) the PCR sample may be immediately heated to 100°C for 3 minutes then plunged into ice where it remains for 4 minutes. This rapid cooling favours annealing of primers to their compliment over annealing of the longer PCR-synthesised DNA to its compliment [*need reference*]. One unit of Klenow DNA-polymerase or T-7 DNA-polymerase is added and incubated at 37°C for 5 minutes to synthesise the newly primed complimentary strand.

Samples that consist of two homoduplexes prepared by this method produce an absorbance versus temperature curve that is the sum of the absorbances of each of the two homoduplex molecules. The melting temperature of polymorphic melting-domains will differ between each molecule. Assuming that the concentration of each homoduplex is slightly different, the relative absorbance of each can be determined from the curve. The relative increase in absorbance with denaturation of analogous polymorphic melting domains, equals the relative absorbance of each molecule. By subtracting the absorbance due to one homoduplex from the absorbance versus temperature curve we can find the absorbance versus temperature curve of the other. The same principle can be applied to composite curves for heteroduplex PCR-amplified products.

The method of the present invention has applications in diverse fields, including systematics and population studies, medical genetics, biodiversity assays, microbial pathology, forensic science and thermal characterisation of microsatellite allomorphs to determine relatedness. Detection of single-base changes using the method of the present invention would facilitate rapid identification of previously characterised mutations simplifying the identification of population-specific allotypes. Point mutations are also responsible for

"carrier" status in many genetic diseases. The automated detection of allotypes using allele-specific PCR-amplification and the method of the present invention offers an alternative to more labour-intensive electrophoretic techniques used for screening. Another area in which the method of the present invention has immediate application is HLA typing.

In another aspect of the invention there is provided a method of comparing DNA including:

providing a source of DNA for comparison;

subjecting the source of DNA to a pre-determined temperature sufficient to cause a portion of the DNA to denature or to renature;

determining the optical density of the DNA at the pre-determined temperature; and

comparing the optical density of the DNA to the optical density of another source of DNA which has undergone similar temperature conditions.

Individual samples can be measured at a pre-determined denaturing or renaturing temperature and the optical density, will be dependent on the complex intermolecular and intramolecular interactions governed by the composition and order of nucleotides within the dsDNA. Small changes in the nucleotide sequence will affect the denaturing or renaturing of the DNA which will be reflected in the optical density by virtue of the proportion of the DNA denatured or renatured at a particular temperature.

Similar temperature conditions include conditions of concentration, temperature, and processing which are comparable or can be standardized so that a direct comparison between samples can be made.

In a further aspect, the present invention provides a method of comparing DNA including:

providing a source of DNA for comparison;

subjecting the source of DNA to one or more temperatures;

determining the optical density of the DNA at one or more temperatures to provide a denaturing profile;

comparing the denaturing profile of the DNA to the denaturing profile of another source of DNA which has undergone similar temperatures.

Specific applications for comparing DNA include detecting single base substitutors between mutant and wild-type strains; detecting mutants in general by measuring subtle changes in T_m; conducting phylogenetic analysis for determining different strains such as viral strains; detecting micro satellite allomorphs for comparing relatedness; HLA typing; use of genetic tags; detecting polymorphic DNA; considering biodiversity and using mitochondrial PCR-amplified products to distinguish populations (Norman et al 1994).

This latter method also could replace sequence-specific oligonucleotide (SSO) probes currently used where population-specific markers are used (see Stoneking *et al* 1991). It could also replace allele-specific oligonucleotide (ASO) probes for detecting disease-specific mutations (Irvanson and Taylor 1991). Using this technique, rapid diagnosis facilitating appropriate application of therapeutic agents would be available Mack et al (1990). This method could readily facilitate population analysis to determine genetic distance and/or genetic diversity within and between populations. Genetic diseases could be efficiently screened for where parental phenotypes might predispose offspring to a particular condition, also allowing reliable chorionic villi sampling (CVS) to be carried out with a short time between sampling and the retrieval of results.

The denaturing profile may be a curve of absorbance change versus temperature or it may be a standardised curve of ΔAbs/Δtemperature Vs. temperature. The curve may indicate denaturing or renaturing temperatures at points where there is a measurable change in absorbance. The change in absorbance may be a sudden increase in absorbance indicating a melting domain. The change is determined by a measurable deviation from the slope of the curve when there is no denaturation of the DNA. The absolute measurable change in absorbance will be depend by the accuracy of the equipment used. Preferably the change will be greater than 0.01 unit absorbance/°C and most preferably the change will be greater than 0.1 unit absorbance/°C.

The denaturation profile may also be used to determine the length of the melting domains.

Other specific uses of the method of the present invention include:

1. Detecting single-base substitutions

Using the method of the present invention, differences as small as a single base change between cloned inserts or PCR products are readily detectable. Changes in dsDNA sequence produce observable changes in T_m (denaturation temperature). Using the method of the present invention these are seen most readily as shifting peak positions when comparing first derivative curves of mutant and wild-type dsDNA (with a defined length). In most point mutations melting-domain length does not change.

2. Detecting sequence mutations in cloned inserts up to 50 kb long. Localisation of base changes to specific restriction fragment is possible with the method of the present invention. Following enzymatic cleavage of long [$\leq$ 100 kb] homogeneous dsDNA with enzymes of restriction sites using double digests is a routine procedure. Cleavage at restriction-sites that lie within a melting-domain will produce two novel melting-domains that when combined will have the same length as their progenitor but each will have a lower melting temperature. Comparing wild type to mutant DNA, shifting peak positions when comparing first derivative curves will localise the mutation to a particular peak. To interpret these peak data a probability algorithm which uses melting-domain length [ls] from the method of the present invention to reconstruct fragment-specific peaks is needed.

3. Phylogenetic analysis using thermal characterisation of full-length mitochondrial DNA.

The potential of the method of the present invention in comparing two long dsDNA molecules with a defined length has numerous applications in population genetics. Mitochondrial DNA (mtDNA) is usually about 16 kb long and can be purified from genomic DNA using density-gradient centrifugation. Phylogenetic analyses using RFLP data from restriction digests of mtDNA are commonplace in the literature. The method of the present invention can be used in a similar manner; the two characteristics, melting-domain length and domain-melting temperature can be used to gather unrefined phylogenetic information. The method of the present invention is not constrained by the

recognition of a specific sequence to be informative of DNA-sequence differences. The method of the present invention in combination with various restriction digests, using the method described in the previous paragraph, would provide better phylogenetic resolution than RFLP analysis alone.

4. Detection and characterisation of microsatellite allomorphs using the method of the present invention.

The discovery of length-specific allelic inheritance of microsatellite loci has been a boon for geneticists mapping genes and to population biologists looking at relatedness between individuals. Two obstacles to fully efficient analysis become apparent when using PCR-amplified microsatellite loci to obtain information in relatedness studies. They are the presence of length homoplasy [the PCR-amplified product from each allele is the same length but not the same sequence] and the time needed for electrophoretic separation of radio-labelled microsatellite PCR-amplified DNA using polyacrylamide denaturing gels. The method of the present invention requires no radiation or electrophoresis and will segregate equal-length DNAs that have different sequences.

5. Thermal denaturation profiles for HLA-typing.

Histocompatability locus antigen (HLA)-typing has dramatically improved with the advent of PCR technology. HLA-typing is done by amplification of variable loci in the HLA gene complex followed by restriction digests using enzyme producing fragments that are then separated using gel-electrophoresis. The digest pattern is HLA-type-specific. It is possible for HLA-matches between donor and recipient - in the case of organ-transplants - to be confirmed or negated within 2-3 hours. Computerised storage of thermal denaturation profiles (TDPs) of donor HLA types would allow donor-recipient matches following PCR amplification of the recipients HLA loci without restriction digests or gel electrophoresis. Typically, a denaturation profile can be obtained in less than 1 hour. If donor-HLA TDPs can be stored on computer then easy comparison between HLA-TDPs with the HLA-TDPs stored and constantly updated at a central location, would facilitate rapid donor-recipient matches unimpeded by the delay imposed by electrophoresis.

6. Thermal denaturation profiles used with genetic tags.

Genetic tags are population-specific sequences usually from maternally-inherited mtDNA that can be used instead of physical tags to distinguish individuals from different source populations. Their practicality exceeds physical tagging systems in that a small tissue or blood sample is all that is required to uniquely identify the population of origin of the sample. They are unaffected by environmental factors and can be identified from tissue or remains where mtDNA can be extracted and amplified - using PCR. In the past either DNA-sequencing or RFLP data has been used to source DNA where the antecedents for genetic tags were known. The method of the present invention does not require electrophoretic resolution of restriction fragments or the technical expertise required for DNA sequencing. After screening extant populations to identify fixed DNA-sequence differences that are population-specific, TDPs for PCR-amplified "tag" product can be used to identify the population of origin of an individual. The method of the present invention can characterise many PCR-amplified "tags" simultaneously with reduced manipulation.

7. Genetic screening using the method of the present invention.

The time-efficient characterisation of polymorphic sequences is an important consideration in screening for genetic diseases. Computerised data collection and centrifugal spectrophotometry facilitate large-sample-size analyses using the method of the present invention to detect polymorphic DNA from many individuals simultaneously. Current methods of genetic screening use the same techniques for molecular characterisation as genetic tags with analogous limitations. The method of the present invention analysis supersedes methods that resolve different DNA sequences using electrophoresis. Comparison of TDPs from STOP DNA analysis with standard TDPs of previously characterised polymorphic sequences separates analysed samples into their various haplotypes.

8. Biodiversity assays.

First derivative curves from the method of the present invention can be used to detect the thermal-fingerprint of a particular PCR-amplified DNA

sequence where other dsDNAs are also present. Using this principle two highly conserved - class-specific - regions flanking a species-specific variable region - in soil bacteria or aquatic microorganisms - could be used to design PCR-primers that amplify the intervening species-specific variable region. Ideally the amplified region would represent one or at most two melting domains, PCR-amplification of a tRNA gene would satisfy both criteria. A series of peaks from first derivative curves, each corresponding to a PCR product-specific domain melting event peculiar to the species of origin will be seen.

9. Microbiological analysis using TDPs.

Thermal denaturation profiles of PCR-amplified dsDNA of strain-specific-DNA sequences and genes coding for antibiotic resistance can be used as references to diagnose bacterial infections and bacterial-specific-therapeutic resistance. With the advent of XL-PCR the amplification of PCR-products as long as 30 kb has been possible, the ability of the method of the present invention to characterise long dsDNA molecules with a defined length means that large portions of bacterial genomes can be screened for polymorphisms, making this method an attractive alternative to the labour intensive isolation of base-changes - that result in novel antibiotic-resistant mutants - using genome-subtraction or DNA-DNA-hybridisation methods.

In another aspect of the present invention, there is provided an apparatus for characterizing DNA including:

a sample chamber for receiving a sample of DNA;

a temperature source for applying one or more temperatures to the sample chamber such that the DNA is subjected to one or more temperatures;

a detector for detecting optical density of the DNA sample;

such that when a sample of DNA is subjected to one or more temperatures, the detector determines a change in optical density as the DNA denatures.

In a further aspect of the invention, there is provided a computer system which analyses the optical density change preferably to further determine the denaturation temperature and the length of the melting domain.

The method may be automated by adopting a spectrophotometer with a heating block for a specific application; automatically increasing temperature, with continuous concurrent readouts of temperature and absorbance. This may produce an easily-interpreted and unique thermal denaturation profile (TDP) for a DNA sample preferably a PCR-amplified DNA of specific sequence and length. The same principle demonstrated above could also provide a thermal renaturation profile (TRP) further enhancing the analytical value of the method.

For genetic studies, large sample sizes are often required to be analysed. To this end, automation of the method described here - with its inherent accuracy and ease of interpretation may accommodate the screening of multiple samples simultaneously. This may be achieved by the samples being loaded into a carousel similar to those used in centrifugal autoanalysis, with the samples heated for instance, by infused air while the temperature in the sample cells is recorded. The absorbance may be read as each cell passes the spectrophotometric detector. Both the temperature and the absorbance data may be collected and stored by computer. For each sample, the continuum may then be plotted. Storage on a computer may also accommodate quick and easy comparison of TDPs and TRPs between even large numbers of samples.

The present invention will now be more fully described with reference to the following examples. It should be understood however that the description following is illustrative only and should not be taken in any way as the restriction on the generality of the invention described above.

In the Figures:

Figure 1 shows a hypothetical plot of absorbance against temperature for a hypothetical PCR-amplified product with domain melting temperatures of 63°C and 78°C, and with a length of 2000 bp.

Figure 2 is a standardised plot showing the slope of the curve in Figure 1 for the hypothetical PCR amplified product showing two melting-domains.

Figure 3 shows a composite of the plots in Figures 1 and 2 with the second abscissa showing the length of the melting-domain.

Figure 4 shows the curve of A260 nm Vs. Temperature for denaturation of a 1050 bp Mitochondrial Cytochrome b gene (Cyt b) of Rhytidnoponera Species 12.

Figure 5 shows the curve produced by standardising A260 nm data from Figure 4 to the range of 0 to 1.

Figure 6 shows the curve of A260 nm Vs. Temperature for denaturation of a Hind III-digested lambda-bacteriophage between the temperatures of 82°C and 88°C.

Figure 7 shows the curve produced by the first derivative of the curve in Figure 6.

Figure 8 illustrates a schematic diagram for a spectrophotometer connected to a computer facility for automation of the STOP DNA analysis.

Figure 9 shows the unstandardised denaturation curve (absorbance versus temperature) curve given by the formula $A_{sample} = fn(t)$ for a hypothetical 2kb PCR product with two melting domains - the first is 412bp melts at 63°C the second is 1588bp and melts at 78°C. The calculations used to standardise this curve and thus determine melting-domain length are shown below. Two assumption are made; 1, that the length of the PCR product is known and that the sample contains only 1 amplified dsDNA species and 2, domain-melting occurs between two consecutive data collection points (in the example data collection points are at 0.1 degrees Celcius intervals).

Figure 10 shows absorbance versus temperature curve after subtracting A_{ds}. the formula for this curve can be given as $A_{sample} = fn(T) - A_{ds}$.

Figure 11 shows the standardise curve of Absorbance versus temperature. The curve in Figure 2 is transformed by multiplying all product-denaturation-specific absorbance data values for a specific denaturation curve by $1/A_{ss}$ for that sample as in equation 11. The formula for this curve is given by $A_{stnd} = R_0 [fn (t) - A_{ds}]$ where $R_0 = 1/A_{ss}$.

Figure 12 shows first derivative curve of a standardised plot for the denaturation of the same hypothetical 2kb PCR-product. The peaks represent specific domain-melting events, the x-coordinate at peak-maxima corresponds

to the temperature at which the rate of denaturation is greatest; the T_m the melting-domain that denatures over this narrow temperature range.

Figure 13 shows the second derivative plot of the same, hypothetical 2kb PCR-product the x-axis intercepts labelled with "■" correspond to the x-coordinate at peak-maxima seen in Figure 4, or the T_m of that particular melting-domain.

Example 1 - Characterisation of DNA

The present invention defines a method of characterising DNA by detecting base changes to measure length of melting domains [l_s] and to determine denaturing temperatures [T_m]. The method includes providing a source of DNA; and measuring the change in optical density of the DNA at varying temperatures. These changes in absorbance can characterise double-stranded DNA using the following calculations:

Let:

σ = fraction of dsDNA of defined length that is single-stranded at a specific temperature

σ_n = fraction of dsDNA that becomes single-stranded as the *n*th domain melts

l_s = length of dsDNA that is single-stranded at a specified temperature (bp)

l_p = length of dsDNA (bp)

A_{ds} = Absorbance of double-stranded DNA before denaturation (absorbance units)

A_{ss} = Absorbance of the DNA when it is fully denatured (absorbance units)

A_{sample} = measured absorbance of sample (absorbance units)

A_{stnd} = standardised absorbance of sample at denaturation (absorbance units)

All absorbance values taken at wavelength 260 nm while temperature increases

Then for a homogenous dsDNA molecule of length l_p.

$$A_{sample} = A_{ds}(1-\sigma) + A_{ss}(\sigma) \quad \text{at any temperature} \qquad (1)$$

therefore

$$\sigma = \frac{A_{sample} - A_{ds}}{A_{ss} - A_{ds}} \qquad (2)$$

The general formula for the *n*th melting domain is therefore given by

$$\sigma_n = \frac{A_{sample} - A_{ds}}{A_{ss} - A_{ds}} - \sum_{1}^{n-1} \sigma \qquad (3)$$

This allows calculation of the length of the *n*th melting domain as follows:

$$l_s = \sigma_n l_p \qquad (4)$$

A correction factor; R_0 compensates for variation in concentration between samples. Using one of the samples as the standard, the factor R_0 is defined as

$$R_0 = \frac{(A_{ss} - A_{ds})_{standard}}{(A_{ss} - A_{ds})_{sample}} \qquad (6)$$

Therefore, the standardised absorbance is given by

$$A_{stnd} = R_0 \times A_{sample} \text{ [at a given temperature]} \qquad (7)$$

For easy comparison of curves between different samples, absorbance (A_{sample}) versus temperature (T) plots can be standardised. Using the formula for absorbance as a function of temperature

$$A_{sample} = fn(T) \text{ [figure 1]}$$

for standardised curves

$$A_{stnd} = R_0(fn(T) - A_{ds}) \qquad (8)$$

In order to plot standardised curves of absorbance (A_{sample}) versus temperature (T) data, the range of the absorbance for the sample should be between 0 and 1. This is achieved by settling the absorbance value(s) for each sample before denaturation to zero as follows.

Let

$$A_{ds}(\text{sample}) = 0 \qquad (9)$$

equation (3) then becomes

$$\sigma_n = \frac{A_{sample} - A_{ds}}{A_{ss}} - \sum_{1}^{n-1} \sigma \qquad (10)$$

and when 1 is substituted for A_{ss} of the standard in equation (6)

$$R_0 = \frac{1}{A_{ss}(\text{sample})} \qquad (11)$$

equation (8) becomes

$$A_{stnd} = R_0[fn(T)] \qquad \text{[Figure 2]} \qquad (12)$$

From plots of standardised data, it can be shown that

$$\sigma_n = A_{stnd(n)} - A_{stnd(n-1)} \qquad (13)$$

The denaturation temperature (T_m) of specific melting domains may then be calculated to indicate the temperature corresponding to the maximum rate of denaturation for that melting domain. The plot of the first derivative of standardised absorbance versus temperature data reveals a series of peaks.

Figure 1 shows a hypothetical plot of absorbance against temperature for a hypothetical PCR-amplified product with domain melting temperatures of 63°C and 78°C, and with a length of 2000 bp.

The plot in figure 2 is of the slope of the curve in figure 1, and figure 3 shows is a composite of the plots in figures 1 and 2, with the second ordinate (y) axis showing the length of the domains. The calculations below give the equations used to determine the length of domains, l_{s1} and l_{s2}.

In Figure 1, it can be seen that three plateaux occur, labelled A, B and C, representing absorbance values under the following conditions:

At A, $\sigma = 0$ and $\delta = 1$

At B, $\sigma = 1 - \delta$

At C, $\sigma = 1$ and $\delta = 0$

From the figures it can be seen that:

Absorbance at B = 0.4 (absorbance units)

$T_{m1} = 63°C$

$T_{m2} = 78°C$

$A_{ds} = 0.33$ (absorbance units)

$A_{ss} = 0.50$ (absorbance units)

Substituting these values into equation (2) gives

$$\sigma = \frac{0.4 - 0.33}{0.50 - 0.33}$$

$$= 0.421$$

$$l_{s1} = \sigma \times l_p$$
$$= 0.412 \times 2000 \text{ bp}$$
$$= 824 \text{ bp}$$

$$l_{s2} = (1 - \sigma) \times l_p$$
$$= (1 - \sigma) \times 2000 \text{ bp}$$
$$= 1176 \text{ bp}$$

A plot (Figure 2) of $\Delta Abs/\Delta T$ against T shows peaks corresponding to the T_m of the domains (l_{s1} and l_{s2}), the corresponding peaks in the plot of $\Delta Abs/\Delta T$ and domain length versus T characterise both the unique T_m and length of the domains (Figure 3).

Example 2 - Characterisation of a Mitochondrial Cytochrome b gene (cytb) of Rhytidnoponera Species 12

A 1050 bp PCR product containing the mitochondrial cytochrome b. gene (cyt b.) of *Rhytidnoponera species* 12 was prepared using standard PCR techniques. A 100µl PCR of the sample was purified using a Wizard-PCR column® supplied by Promega Corp.

STOP DNA analysis was performed on a Varian 3E® UV-Vis spectrophotometer equipped with Cary® software. The sample was placed in 2.9ml of water in a 3ml cuvette supplied by Starna Co. Pty. Ltd (Sydney, Australia) and heated at 1°C/min using a Varian® temperature controller accessory. Absorbance readings at 260 nm were collected by the computer at 0.1°C intervals with the signal band width on the spectrophotometer set at 2.0 nm. Figure 4 shows the curve of A260 versus temperature for denaturation of the 1050 bp PCR product.

Figure 5 shows the curve produced by standardising A260 data from figure 4 to the range 0 to 1 using equations 9-12 substituting for A_{ds} = 30.66 and A_{ss} = 13.48.

Melting domain lengths and corresponding denaturation temperatures of the 1050 bp PCR product from Rhytidnoponera species 12 were then calculated and are shown in Table 1. These values are taken from the standardised curve (figure 5) and the maxima values from the first derivative of absorbance versus temperature respectively (data not shown).

Table 1: Melting Domain Lengths and corresponding denaturation temperatures or a 1050 bp PCR product from Rhytidnoponera species 12.

Domain number	Fractional denaturation [σ]	Melting-domain length [σ x 1_p]	Domain-melting temperature °C [T_m]
1	0.287	301	52.70 ± 0.05
2	0.510	535	56.07 ± 0.05
3	0.068	71	58.20 ± 0.05
4	0.053	56	68.08 ± 0.05
5	0.026	26	72.42 ± 0.05
6	0.056	59	76.30 ± 0.05

Example 3 - Characterisation of DNA from Hind III-digested Lambda-bacteriophage

A sample of Hind III-digested lambda-bacteriophage (1.25μg/ml) was obtained and subjected to STOP DNA analysis under conditions identical to those described in Example 2. Figure 6 illustrates a plot of A260 versus temperature between 82°C and 88°C for the denaturation of the Hind III-digested lambda-bacteriophage.

Figure 7 shows the first derivative of the plot obtained in Figure 6 and illustrates a plot of Δ-A260/Δ-temperature versus temperature (the first derivative) between 82°C and 88°C for the denaturation of Hind III-digested lambda-bacteriophage. This curve illustrates the resolution possible by using STOP DNA to determine denaturation temperatures for melting domains in large double-stranded DNA molecules.

On the absorbance versus temperature curve each rise in absorbance represents the melting of a paricular domain. A rise is followed by a plateau before the next domain-melting event occurs. The ΔAbs/Δtemperature versus temperature curve shows a set of peaks which correspond to the rises seen in the absorbance versus temperature curve while the zero or minima correspond to the plateaux. It is the x-coordinate's (temperature) value at the peak maxima on the ΔAbs versus Δ temperature curve that represents the greatest

rate of melting of a specific domain designated herein as the melting temperature (Tm) of that domain.

Example 4 - Automation of the STOP DNA analysis

The present method of characterising DNA based on changes in optical density have many advantages over the prior art. Its use requires little technical expertise, significantly reduces handling of samples and generates rapid results which are reproducible and reliable. To facilitate the rapid generation of results, automation of the method is relatively simple. A spectrophotometer with an apparatus for automatically increasing the temperature can be connected to a computer to provide continuous concurrent readouts of temperature and absorbance.

The present example in Figure 8 provides a schematic illustration of a spectrophotometer with labels and computer specifications which can be applied to the present method.

Computer software parameters.

User entry field:

Sample specification; sample name, position in microtitre tray; AH, 1-12, select position (cuvette number) in sample cuvette numbers 1-96 (cell numbers 2-25, 27-50, 52-75, 77-100) or automatic option using automatic computer selection for the best loading format for up to 96 samples including blanks to balance the carousel.

Ramp rate for heating samples; Range from 0.5 to 5 °C/min. The feedback for thermo-electric heated fan is the average of 40 consecutive readings from the four thermistors immersed in an equal volume of buffer identical to the sample buffer in the carousel cartridge at carousel positions l,26, 51, 76. A new afferent temperature point is generated at every 1/4 turn of

the carousel. These temperature points are collected and averaged over 10 revolutions. The average is used as the positive feedback temperature to set the gain for the thermo-electric heating fan. For example, using a centrifugal velocity of 60 rpm and a temperature ramp rate of 1°C/min each cell passes through the light-beam at approximately 0.016 °C intervals (1 time per sec). The feedback temperature, calculated as the average of all four probes over 10 cycles will be approximately +0.16°C/sec.

Data collection and storage: As respective cells pass the origin (position 1) adjacent to the photomultiplier tube, temperature readings from consecutive probe-cells (cells 1 and 26, 26 and 51, 51 and 76, and 76 and I next) and the transmittance of the samples - [Transmittance (emitted)] - intervening each probe cell with the intensity of the uninterrupted beam - [Transmittance (incident)] can be recorded separately. Data points are calculated using the general formula

$$\textbf{Absorbance} = \frac{\sum_{1}^{10}\left[-\log\left[\frac{\text{Transmittance (emitted)}}{\text{Transmittance (incident)}}\right]\right]}{10}$$

Temperature = $\sum_{1}^{10}$ [$T_{cell\ 1}$ + $T_{cell\ 26}$ (for 10 revolutions)] / 20 for sample cells 2 to 25

Temperature = $\sum_{1}^{10}$ [$T_{cell\ 26}$ + $T_{cell\ 51}$ (for 10 revolutions)] / 20 for sample cells 27 to 50

Temperature = $\sum_{1}^{10}$ [$T_{cell\ 51}$ + $T_{cell\ 76}$ (for 10 revolutions)] / 20 for sample cells 52 to 75

10

Temperature = $\sum_{1}$ [Tcell 76 + $T_{cell\ 1[new]}$ (for 10 revolutions)] / 20 for sample cells 77 to 100

Data points are then stored in data arrays as (A260, Temperature) values every 10 cycles for each sample cell . First derivative data can be calculated for each sample from these stored values as:

$((A260_n - A260_{[n-1]}) / (T_n - T_{[n-1]}), Temperature_n)$
$(\Delta A260/\Delta T , T)$

Data can be plotted as A260 versus temperature or for first derivative $\Delta A260/\Delta T$ versus temperature in the *result field*. Plots for individual samples can be recalled using their identifier code.

Standardisation of plots as described in "Spectrophotometric typing of polymorphic DNAs (STOP DNA) analysis: a novel technique for molecular analysis." will allow computerised comparisons between sample and selected, stored curves.

User result field:

For each cell, computer software will allow; selection of first derivative $\Delta A260/\Delta T$ versus temperature curves or standardised or non-standardised outputs of A260 versus temperature, on-screen cursor control to extract useful data points from these curves to the *reports page* as well as comparison of standardised curves between samples and stored standards. Subtraction from absorbance versus temperature curves of unwanted plateaux due to heteroduplex, primer dimer or allomorphs would be another useful feature as would computerised determination of T_m from first derivative peaks and melting-domain length from result curves and storage of these values for comparison. Fuzzy logic would provide the necessary "wobble" for criteria of alike-unlike judgements.

Unique Features Of This Spectrophotometer

1. UV absorbance measurements in a centrifugal format.
2. Mathematical interpolation of temperature increments allowing temperature data intervals of 0.16 °C (or as small as 0.016°C depending on variation carousel speed and rate of temperature increase).
3. Recirculation of heated air from an electronically-controlled heating fan integrated into the airflow chamber instead of a heating element.
4. High resolution of T_m and melting-domain length necessary for *STOP DNA* analysis.
5. Application of *STOP DNA* analysis for multiple samples using dedicated software.
6. Temperature monitoring from within a moving cuvette using a transducer that transmits to a stationary receiver on the spectrophotometer chassis.
7. Heat-conductance isolated to that portion of the cuvette that contains sample.

Figure 8 illustrates a schematic diagram for a spectrophotometer with labels and computer specifications which can be used in the present invention.

Labelled features of the schematic representation of a centrifugal spectrophotometer to be used for *STOP DNA* analysis.

In Figure 8 the following apply:

A. Photomultiplier tube
B. Biconcave lens
C. Biconvex lens
D. Diffraction grate and signal-slit assembly
E. Deuterium light-source
F. Beam light-source
G. Cowled heating fan for steady air circulation
H. Thermo-electric control to heat fan
I. Fan and carousel drive-motor

J. Lid assembly

K. Cuvette assembly

L. Insulating ring between the upper and lower sections of the heating fan

M. Carbon-brush contacts for electronic-controlled current supply to heat the fan

N. Thermistor wire to transducer chip (power pick-up for the chip is through carbon brush contacts to conductors that encircle the base chassis as shown in the schematic). Output is digitised and transmitted to receiver (S).

O. Photovoltaic cells that switch input from PM tube to incident or emitted intensity depending on the carousel position.

P. Independent LED's to activate photovoltaic cells (U).

A. Origin indicator (x1 at position 1)

B. Cuvette indicator $4mm^2$ (x 100 positions 1-100 conincident with the centre of the sample)

C. Incident beam indicator $4mm^2$ (x 100 positions 1-100 coincident with the centre of the interrupter)

Q. Carousel bearing

R. Transducer leads for temperature information and electronic-temperature-control feedback.

S. Temperature-transducer radio-receiver

T. Photovoltaic cells for beam-switching

U. Vanes to drive air past the cuvettes

V. Quartz windows for unimpeded UV-light passage

NB. Thin arrows show the direction of airflow the thicker ones indicate carousel rotation

Features that have not been included in the schematic representation.

1. Automated sample loading for up to 96 samples.
2. Computer software utilities:
   a. Selection of temperature ramp rate.

b. Choice of sample order (to be loaded) from a microtitre-plate.

c. Name or identifier of each sample displayed with each sample's temperature denaturation profile.

d. Automatic balancing of the carousel.

e. Selectable speed of the carousel giving variable data intervals (degrees/cycle).

f. User-result interface for data manipulation.

g. Storage of each cell's absorbance versus data in its raw form (transmittance versus temperature) in data arrays for each cell.

h. Long-term computer storage of selected profiles.

i. Selection of up to four temperature probes with the ability to graduate temperature readings over 24 cells.

j. Selection of temperature range (20 to 100 degrees Celsius).

A second spectrophotometer model with 20 cells, two opposed temperature-probes and manual rather than automatic loading would provide an alternative machine at much less expense that operates on the same principle as outlined here.

Example 5

The nature of any data collection method where two parameters [in this case absorbance and temperature] are measured can only be as accurate as the smallest division used to measure a particular parameter, usually the value is taken as the closest whole division ± 0.5 of the smallest division. Thus where a graph is drawn using data obtained from a so called continuous collection it will always be drawn from a series of data points.

A sample of dsDNA of known length in aqueous solution is subjected to a slow increase in temperature. As the temperature increases the DNA denatures in sequence-specific blocks (these are known as melting-domains), producing a quantifiable change in the amount of light that is absorbed at wavelength 260nm.

The absorbance (A_{260}) and the temperature of the sample are recorded as the temperature increases. The temperature difference between the melting temperatures of two analagous melting-domains - in a dsDNA-product of known length where specific base changes have resulted in an altered melting temperature of the melting-domain containing the mutation - may be small. To detect the difference the smallest data increment (temperature increase) must be less than 0.5x the difference between the melting temperatures of the mutant and reference molecule. Current methods (DGGE and TGGE) can detect differences as small as 0.5°C the method of the current invention should be able to detect differences as small as 0.008°C depending on carousel speed and rate of a temperature increase.

A limitation in comparing dsDNA molecules from divergent species using STOP DNA is that two very different DNAs may by chance have identical TDPs. By using small temperature-data collection intervals this will be much less likely to occur.

As can be seen in the additional plots and formulae, data converted to first and second derivative allows rapid determination of the melting temperature(s) of melting-domains from their raw TDP data. Standardisation of the TDP allows the values for σ (the fractional denaturation) to read directly from the standardised denaturation profile [$A_{stnd} = R_o (fn(t) - A_{ds})$].

Finally it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

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CLAIMS:

1. A method of characterising DNA including:

providing a source of DNA;

subjecting the DNA to a range of temperatures sufficient to cause a portion of the DNA to denature or to renature; and

determining the optical density of the DNA within the range of temperatures.

2. A method according to claim 1 wherein the range of temperatures includes a denaturation or renaturation temperature indicating a melting domain in the DNA.

3. A method according to claim 1 or 2 wherein the determination of optical density is measured as a change in absorbance at a predetermined wavelength.

4. A method according to any one of claims 1 to 3 wherein the optical density is measured as a change in absorbance or emission of light as incorporation or dissociation of interchelating agents to quantify the length of DNA that becomes single stranded as dsDNA denatures.

5. A method according to any one of claims 1 to 4 wherein the optical density is measured at a wavelength of about 250 to 300 nm.

6. A method according to any one of claims 1 to 5 wherein the DNA is subjected to more than one denaturation or renaturation temperature range to indicate more than one melting domain.

7. A method according to any one of claims 1 to 6 wherein the temperature range is varied in a continuous or stepwise manner.

8. A method according to claim 7 wherein the temperature is increased by less than 0.1°C/min.

9. A method according to any one of claims 1 to 8 wherein the determination of optical density is measured as a ratio of the change in absorbance and a change in temperature over a temperature range.

10. A method of characterising DNA including:

providing a source of DNA by PCR amplification;

subjecting the PCR amplification to conditions favouring homoduplex DNA molecule formation;

subjecting the DNA to a range of temperatures; and

determining a change in optical density of the DNA over the range of temperatures.

11. A method of comparing DNA including:

providing a source of DNA for comparison;

subjecting the source of DNA to a range of temperatures including a denaturing or renaturing temperature;

determining the optical density of the DNA over the range of temperatures to provide a denaturing or renaturing profile;

comparing the denaturing or renaturing profile of the DNA to the denaturing or renaturing profile of another source of DNA which has undergone similar conditions.

12. A method according to claim 11 wherein said method is applied to any one selected from the group including detecting mutants by measuring changes in T_m; conducting phylogenetic analysis for determining different strains such as viral strains; detecting micro satellite allomorphs for comparing relatedness; HLA typing; use of genetic tags; detecting polymorphic DNA; considering biodiversity and using mitochondrial PCR-amplified products to distinguish populations.

13. A method according to claim 11 or 12 wherein the denaturing or renaturing profile is a curve of absorbance change versus temperature or a standardised curve of ΔAbs/Δtemperature Vs. temperature.

14. A method according to any one of claims 1 to 13 wherein a melting domain is indicated by a sudden change in absorbance.

15. An apparatus for characterising DNA including:

a sample chamber for receiving a sample of DNA;

a temperature source for applying one or more temperatures to the sample chamber such that the DNA is subjected to one or more temperatures;

a detector for detecting optical density of the DNA sample;

such that when a sample of DNA is subjected to one or more temperatures, the detector determines a change in optical density as the DNA denatures or renatures.

16. An apparatus according to claim 15 further including a computer system which analyses the optical density change to further determine the denaturation or renaturation temperature and the length of the melting domain.

17. An apparatus according to claim 15 or 16 wherein the method is automated by adopting a spectrophotometer with a heating block for a specific application; automatically increasing temperature with continuous concurrent readouts of temperature and absorbance.

18. A method according to claim 1 substantially as hereinbefore described with reference to the examples.

19. An apparatus according to claim 15 substantially as hereinbefore described with reference to Example 8.

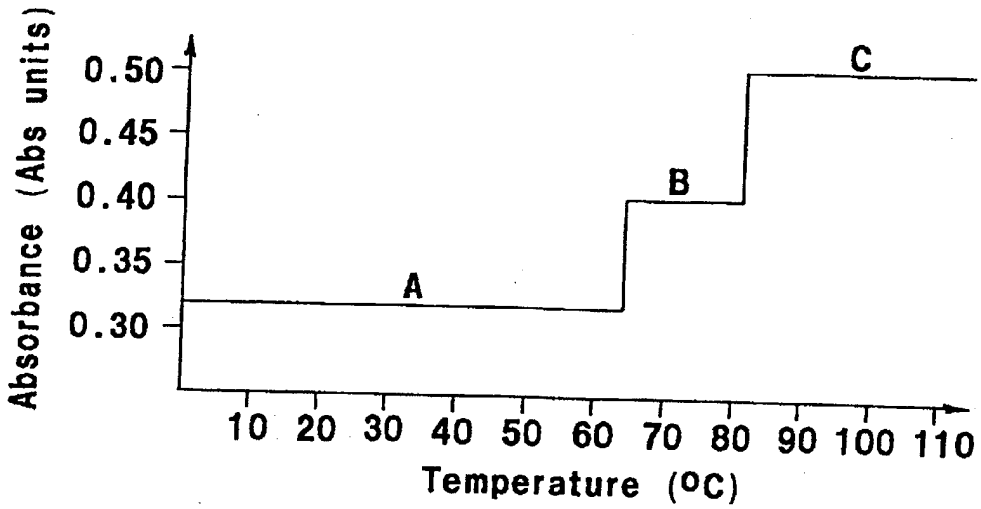
Absorbance (Abs units)
0.50
0.45
0.40
0.35
0.30
A
B
C
10 20 30 40 50 60 70 80 90 100 110
Temperature (°C)

FIG 1

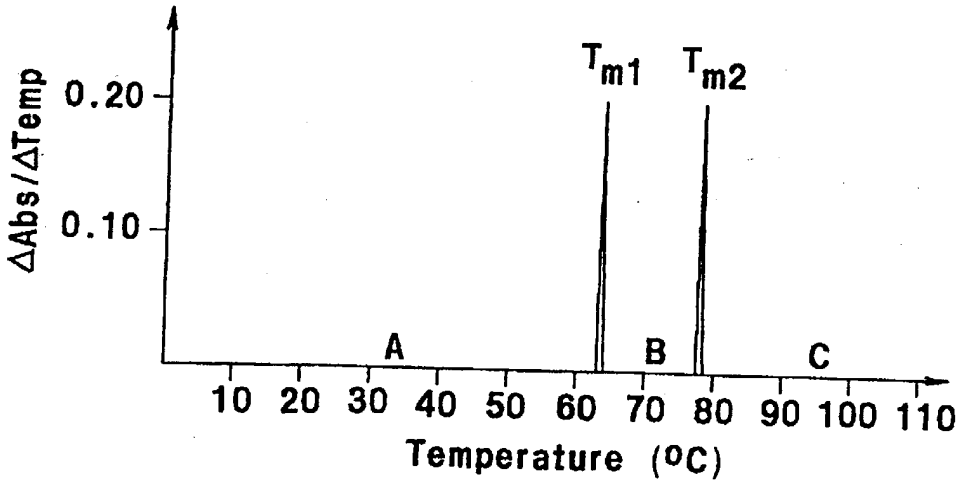
ΔAbs/ΔTemp
0.20
0.10
T_{m1}
T_{m2}
A
B
C
10 20 30 40 50 60 70 80 90 100 110
Temperature (°C)

FIG 2

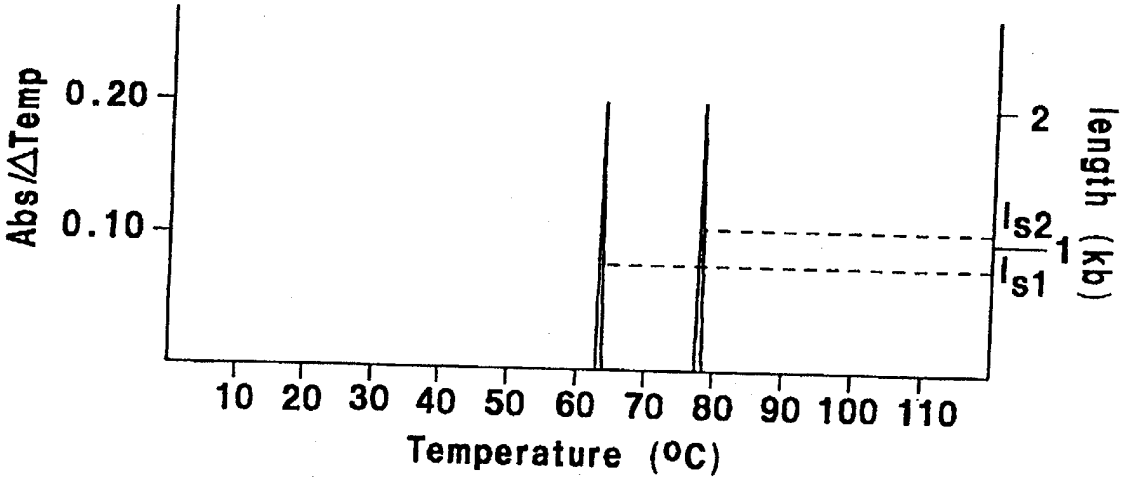
Abs/ΔTemp
0.20
0.10
10 20 30 40 50 60 70 80 90 100 110
Temperature (°C)
2
1
l_{s2}
l_{s1}
length (kb)

FIG 3

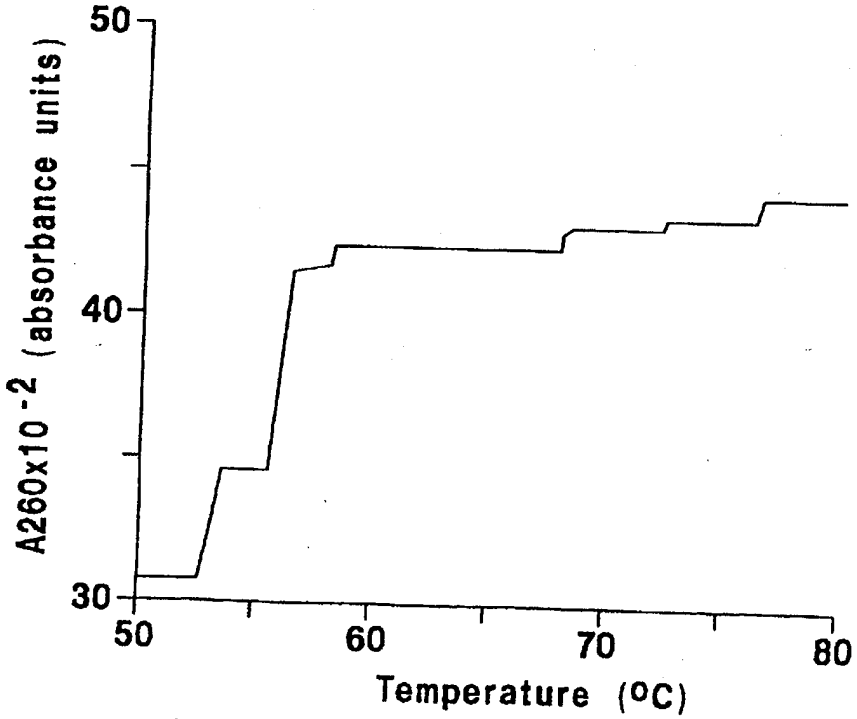
A260x10 -2 (absorbance units)
50
40
30
50
60
70
80
Temperature (°C)

FIG 4

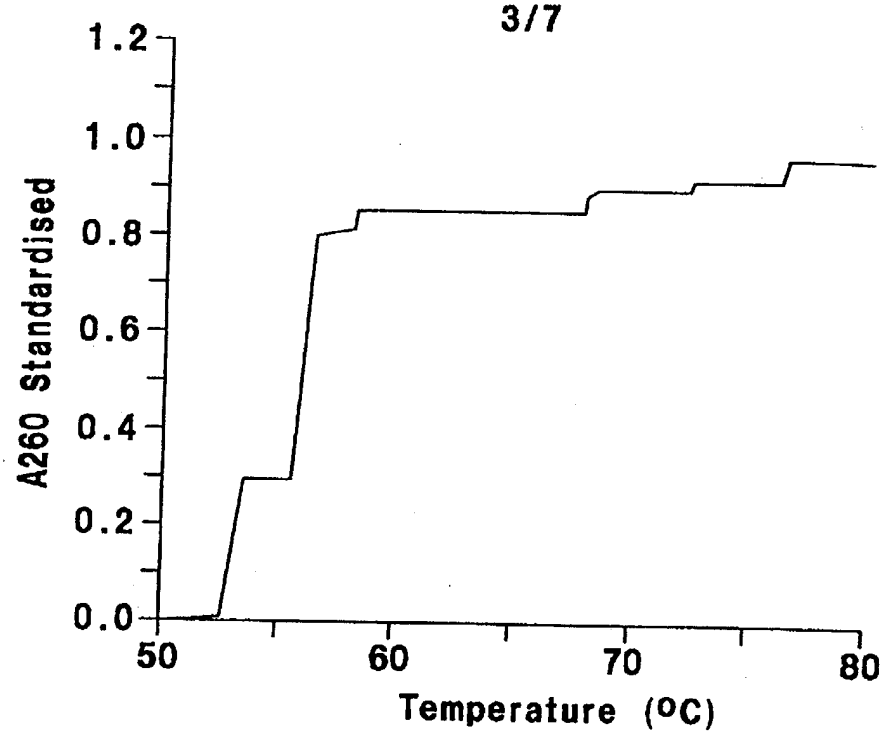
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A260 Standardised
50
60
70
80
Temperature (°C)

FIG 5

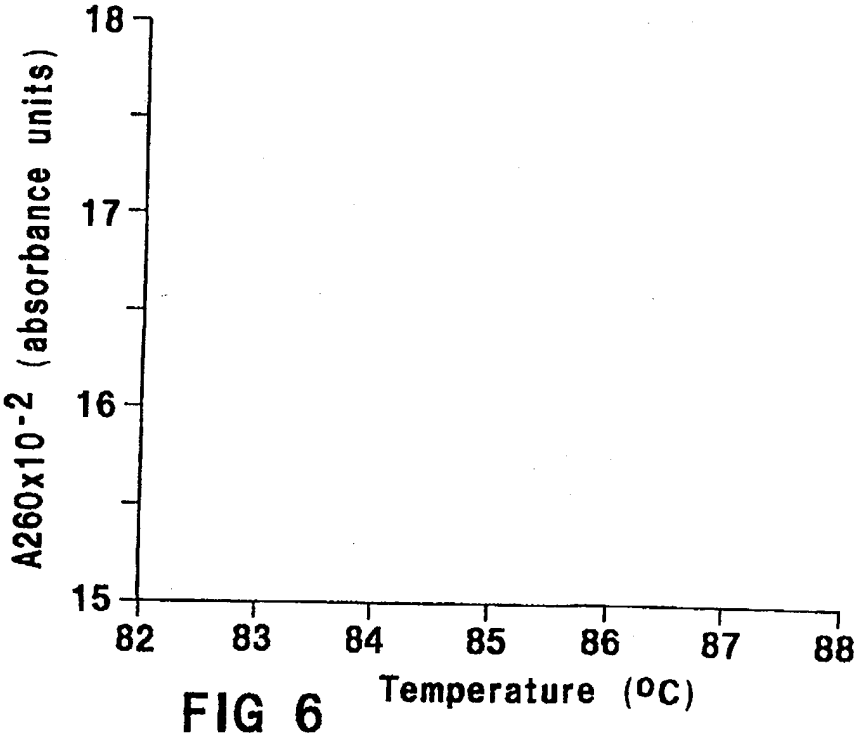
18
17
16
15
A260x10-2 (absorbance units)
82
83
84
85
86
87
88
Temperature (°C)

FIG 6

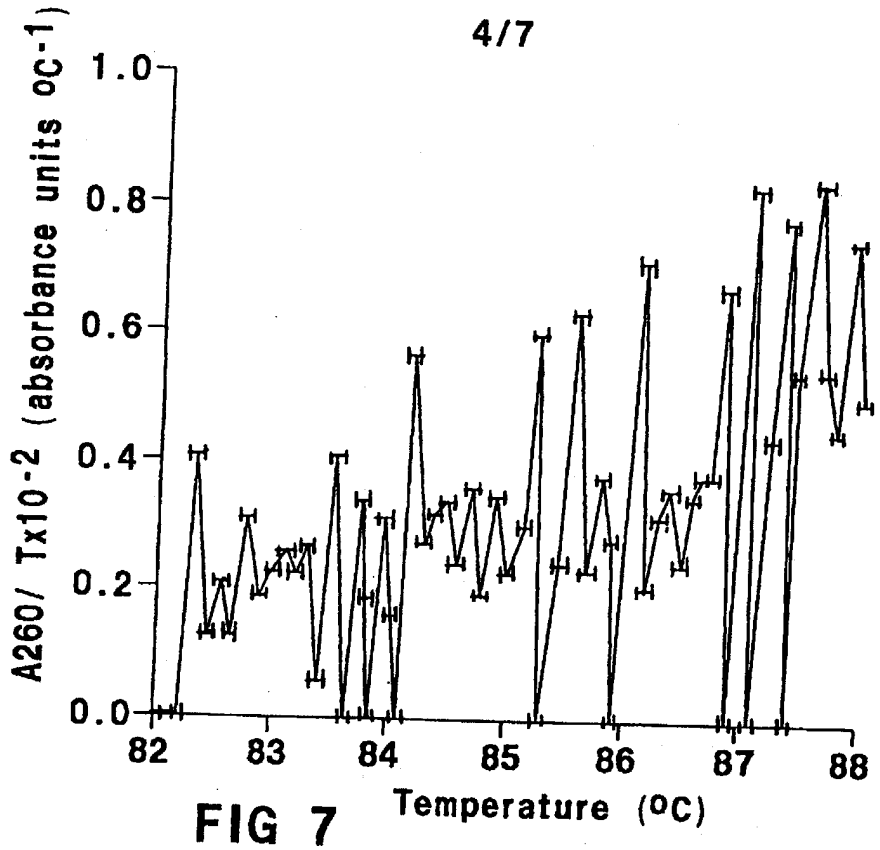
A260/ Tx10-2 (absorbance units oC-1)
1.0
0.8
0.6
0.4
0.2
0.0
82
83
84
85
86
87
88
Temperature (oC)

FIG 7

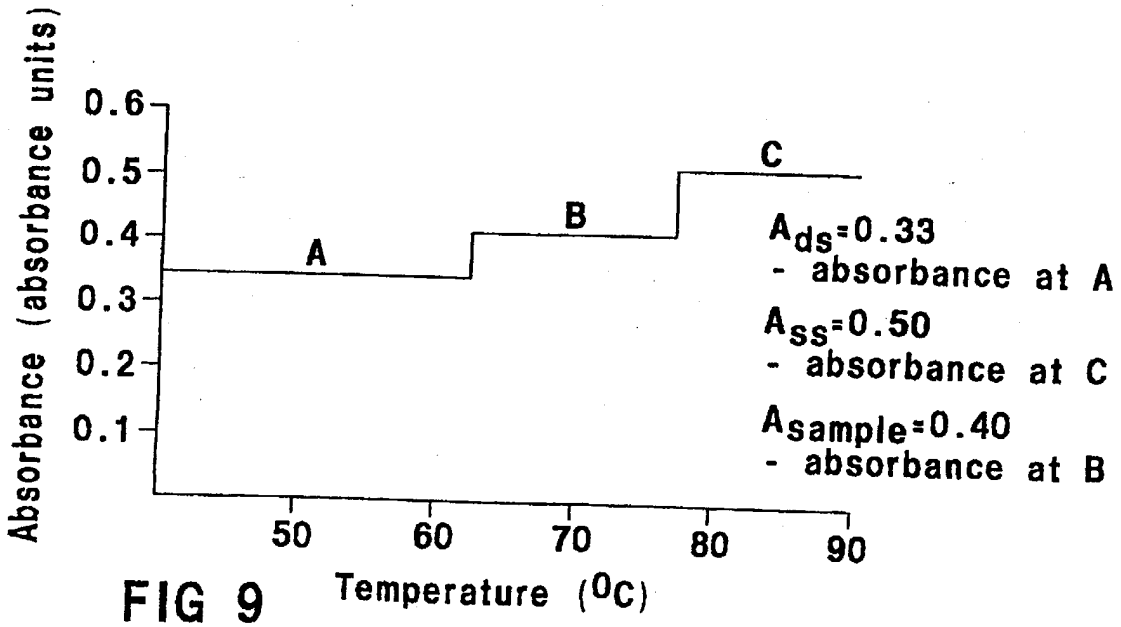
Absorbance (absorbance units)
0.6
0.5
0.4
0.3
0.2
0.1
A
B
C
Ads=0.33
- absorbance at A
Ass=0.50
- absorbance at C
Asample=0.40
- absorbance at B
50
60
70
80
90
Temperature (0C)

FIG 9

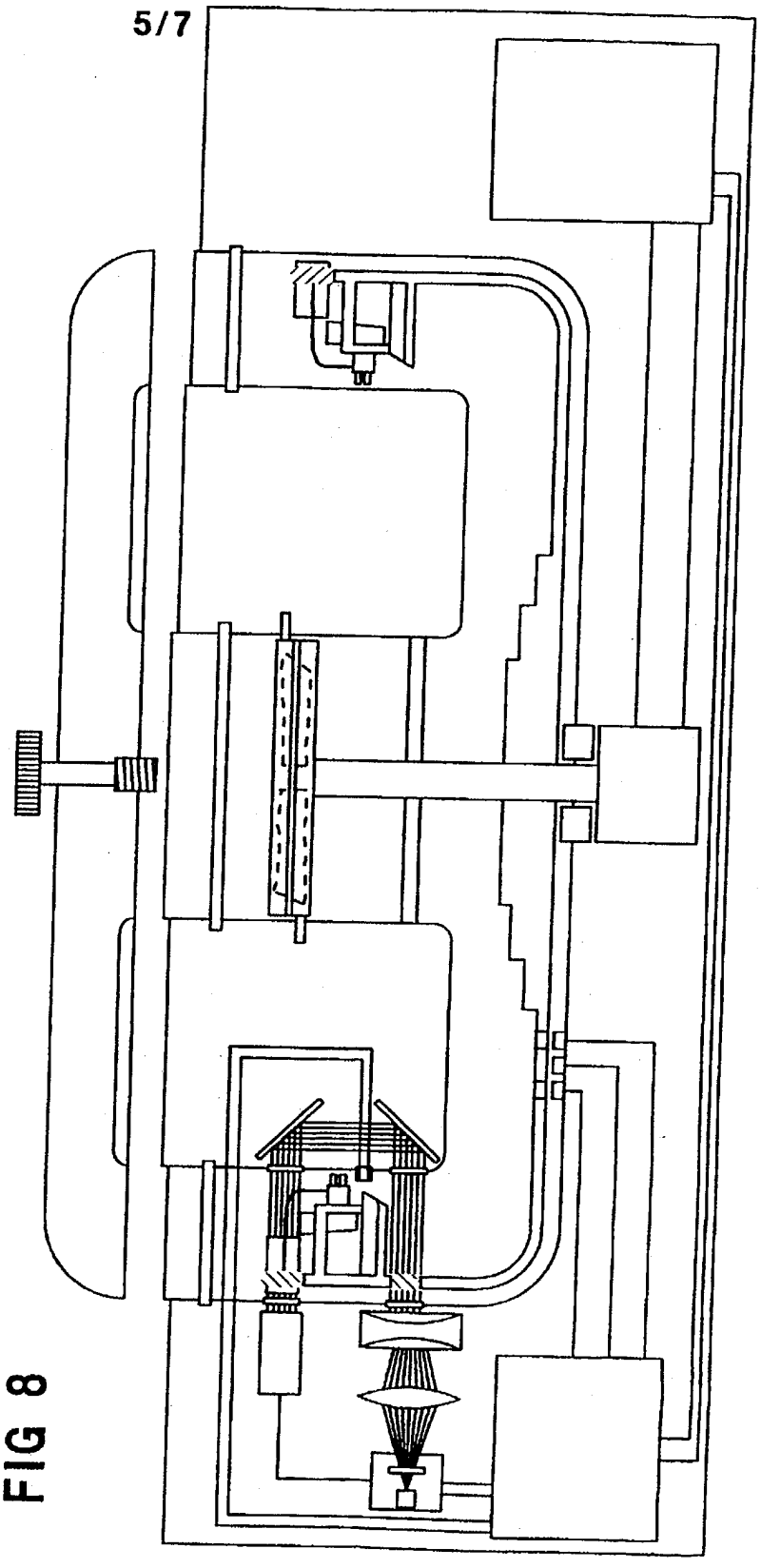

FIG 8

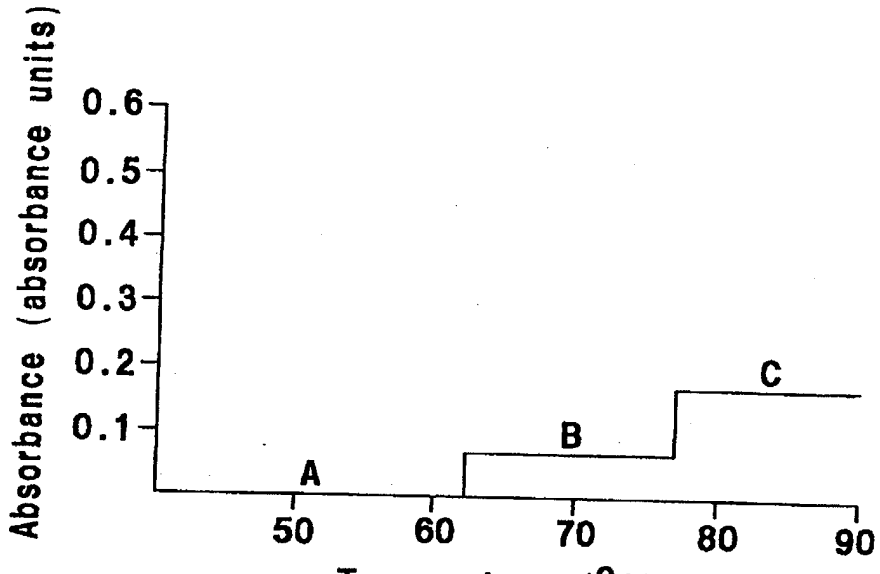
Absorbance (absorbance units)
0.6
0.5
0.4
0.3
0.2
0.1
A
B
C
50
60
70
80
90
Temperature (0C)

FIG 10

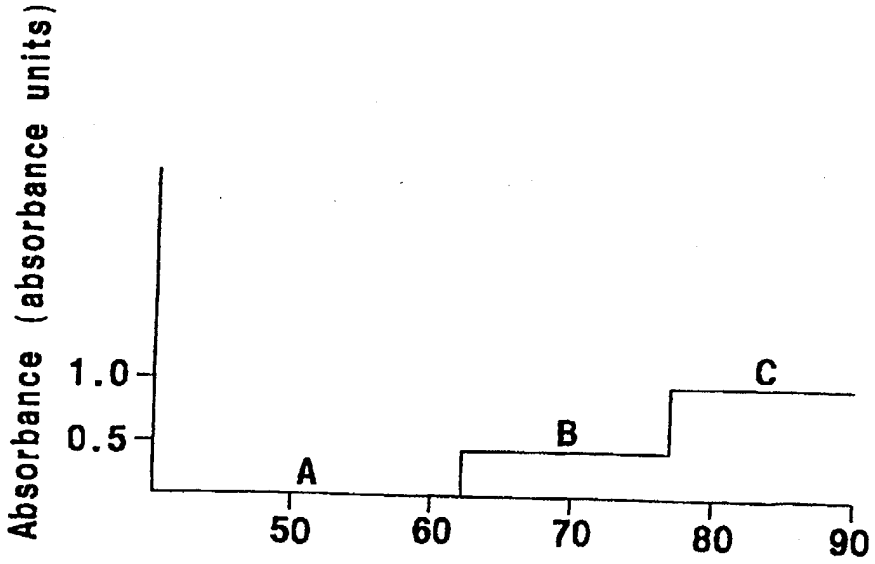
Absorbance (absorbance units)
1.0
0.5
A
B
C
50
60
70
80
90
Temperature (0C)

FIG 11

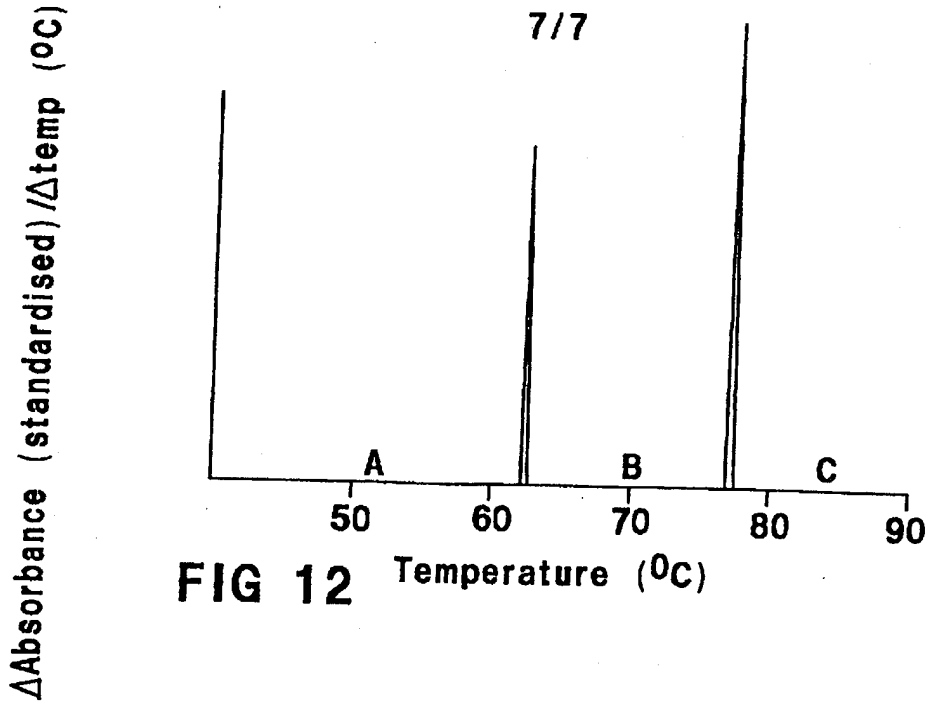
ΔAbsorbance (standardised)/Δtemp (°C)
A
B
C
50
60
70
80
90
Temperature (°C)

FIG 12

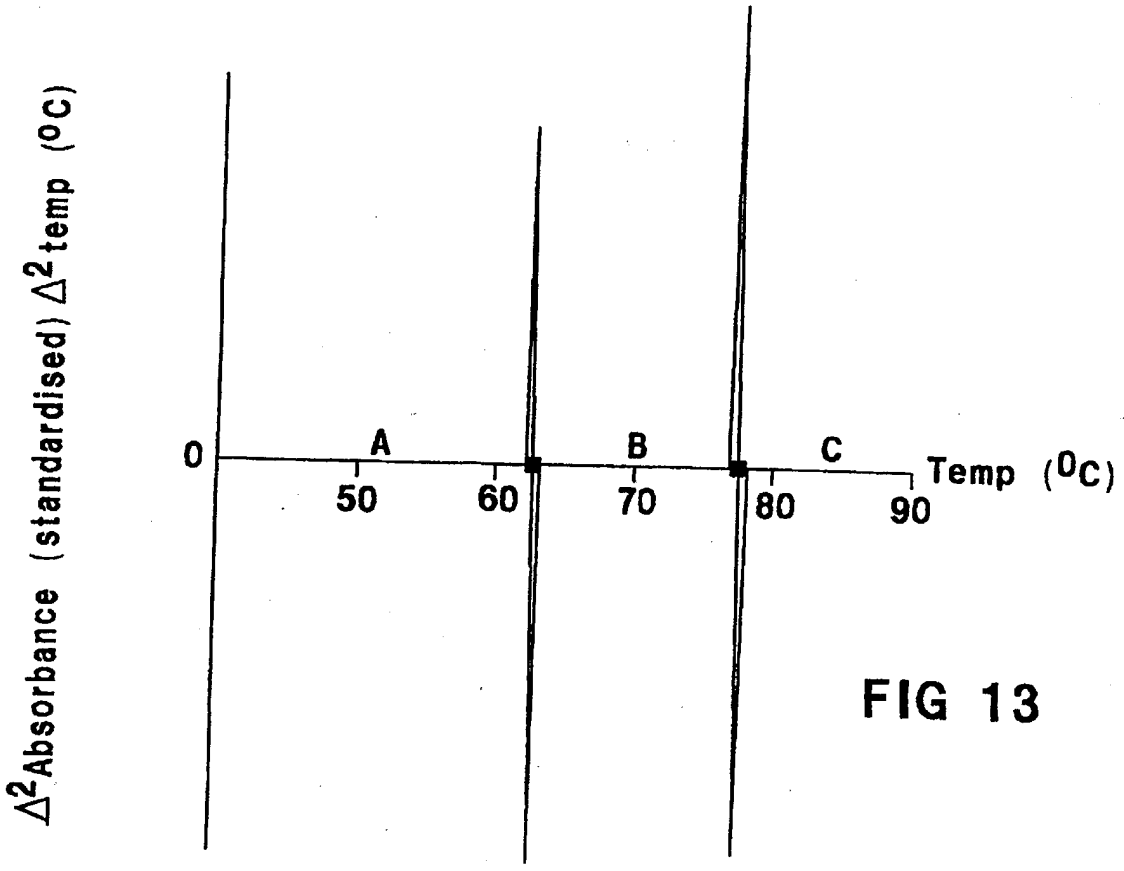
Δ²Absorbance (standardised) Δ²temp (°C)
0
A
B
C
50
60
70
80
90
Temp (°C)

FIG 13

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00595

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/483; C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC:G01N 33/48, G01N 33/49, C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU:IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT:DNA, nucleic acid, absorbance, optical density
STN CAPLUS:DNA/ANST, absorbance, optical density, optical absorption, mutation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0711840 A2 (HITACHI, LTD) 15 May 1996 entire document	1-19
X	WO 96/09532 A1 (ABBOTT LABORATORIES) 28 March 1996 pages 4-5, 8; examples 4-8	1-19
P,X	Derwent Abstract Accession No 97-045827 Class S03, JP,A, 8298998 (Suzuki KK) 19 November 1996 entire abstract	1-19

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 October 1997

Date of mailing of the international search report
29 OCT 1997

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 97/00595

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemistry Vol.16 No.18 (1977) "Comparative Studies on the Secondary Structure of Ovalbumin Messenger RNA and its Complementary DNA Transcript" Ngugen, T. et al. pages 4090-4100 entire document	1-19
X	Biopolymers Vol.19 No.3 (1980) "Analysis of High-Resolution Melting (Thermal Dispersion) of DNA. Methods" Yen, W.S. et al. pages 681-700 entire document	1-3,5-19
X	Biochimica et Biophysica Acta Vol.518. No.2 (1978) "Spectral Analysis & High Resolution Direct-Derivative Melting Cures of DNA for Instantaneous and Total Base Composition" Blake, R.D et al. pages 233-246 entire document	1-3,5-19
X	Biopolymers Vol.13 No.12 (1974) "Determination of the Rate of Renaturation of Modified DNA by Fluorescence Depolarization" Miller et al. pages 2545-2551 entire document	1-11,15-19
X	Biochemistry Vol.33.No.32 (1994) "Melting and Premelting Transitions of an Oligomer Measured by DNA base Fluorescence and Absorption" Xu, D. et al. pages 9592-9599 entire document	1-3,5-11,15-19
X	Nucleic Acids Research Vol.18.No.16 (1990) Thermal denaturation profiles and gel mobility shift analysis of oligodeoxynucleotide triplexes Shea, R.G et al. pages 4859-4866 entire document	1-3,5-11,15-19
X	"Absorption Spectroscopy" in Physical Biochemistry Freifelder, D. 2nd ed. (1982) W.H.Freeman & Company, San Francisco, pages 494-536 pages 507-511	1-3,5-10,15-19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 97/00595

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
EP 711840	JP 8187098
WO 9609532	AU 36362/95 CA 2197321 EP 783683 US 5599668

END OF ANNEX